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CO_2 Excretion Across Isolated Perfused Crab Gills: Facilitation by Carbonic Anhydrase[1]

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SYNOPSIS. Crab gill carbonic anhydrase is shown to facilitate the excretion of carbon dioxide across isolated perfused gills. A technique for perfusing crab gills and assessing the metabolic viability of perfused gills is also described in detail. The technique is used to follow the disappearance of ^{14}C label as HCO_3^- and CO_2 from internal perfusate passing through the gill. The excretion of the label increases with the flow rate of the external perfusate across the outside of the gills. The addition of carbonic anhydrase to the internal perfusate results in a two- to fourfold increase in the excretion of label while Diamox (acetazolamide) treatment decreases the excretion of label by half. It is also suggested that carbonic anhydrase, present in muscle tissues of crabs, minimizes the disequilibrium of the hemolymph CO_2 system as metabolically produced CO_2 leaves the tissues and enters the hemolymph. Parallels are drawn between the presence of carbonic anhydrase in the crab gill system and the presence of this enzyme in the respiratory organs of both aquatic and terrestrial animals.

INTRODUCTION

The respiratory organs of many different kinds of aquatic and terrestrial animals share one common feature: they all possess carbonic anhydrase. Carbonic anhydrase (CA) in this tissue can apparently serve several functions, *e.g.*, CO_2 excretion, acid-base regulation and ionic regulation (Effros *et al.*, 1978; Klocke, 1978; Haswell *et al.*, 1980; Henry, 1984). But I suggest that the ubiquity of the enzyme in respiratory organs of different animals is best explained by its facility in mobilizing bicarbonate from blood or hemolymph into the respiratory epithelium as CO_2 where it can then be used for purposes of acid-base or ionic regulation or excreted into the ambient medium.

A survey of the functions of CA in different groups of animals is consistent with this idea. CA is implicated in ionic regulation only when the respiratory organ is a gill and only when the gill is in contact with the aqueous medium. Fish gill CA, for example, is important in both ionic and acid-base regulation (Haswell *et al.*, 1980). These authors also argue that the large blood bicarbonate pool has access to CA in the gill epithelium. The catalyzed conversion of bicarbonate to CO_2 on or near the basal membrane, adjacent to the blood, supplies ion pumps on the apical membrane with substrate in the form of CO_2 which is rapidly converted by CA to HCO_3^- and H^+. CO_2 may also simply pass across the apical membrane into the ambient water. The dual role of CA in this case requires the localization of CA in a tissue which is fairly complex, where the dehydration reaction predominates in one part of the tissue and the hydration reaction predominates in another part. While the complex morphology of gill epithelia in fish has been firmly established (see for example Laurent and Dunel, 1980), the localization of CA within the tissue is not certain.

Among the aquatic crustaceans, gill CA also participates in ionic and acid-base regulation (see Henry, 1984, for review). As members of this group evolved toward more terrestrial forms, gills continued to be important gas exchange organs and the importance of CA in CO_2 excretion is more readily apparent (Henry, 1981; McMahon and Burnett, 1981; Randall and Wood, 1981). However, the gills of terrestrial crabs can reclaim salts from urine directed to the gill chamber from the antennal gland (Wolcott and Wolcott, 1982). So even in terrestrial crabs, gill CA may retain a role

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in ionic regulation, but this has yet to be demonstrated. As in the case of fish, crab gill CA is implicated in CO_2 excretion even when the gills are continuously immersed in the ambient medium. In a study by McMahon *et al.* (1984) a crab which is capable of little or no ionic regulation was treated with the CA inhibitor Diamox (acetazolamide). Diamox treatment resulted in a significant and persistent elevation of hemolymph P_{CO_2}, a sign of impaired CO_2 excretion.

An interesting example of the involvement of CA in CO_2 excretion in both aquatic and aerial media in a single organism occurs among the air breathing fishes. The lungs of air breathing fishes are gill or opercular elaborations or structures modified from different organs. *Trichogaster trichopterus* is one such air breathing fish whose lungs, called labyrinth organs, derive from the epibranchial regions of the first and second gill arches (Burggren, 1979). *Trichogaster* also possesses gills which it normally uses for CO_2 excretion. Both gills and labyrinth organs possess CA activity. Burggren and Haswell (1979) demonstrated that when the normal path for CO_2 removal by the gills was blocked, the labyrinth organs completely took over the CO_2 excretion function. Furthermore, they demonstrated that CO_2 excretion by the labyrinth organs was inhibited by treatment with Diamox, suggesting that labyrinth CA serves a function in the CO_2 excretion process.

Among the truly terrestrial animals, pulmonary CA plays a similar role in facilitating CO_2 excretion. This phenomenon has been studied intensively in mammals in recent years (Crandall and O'Brasky, 1978; Effros *et al.*, 1978; Klocke, 1978; Effros *et al.*, 1980). A large body of evidence holds that pulmonary CA has direct access to the plasma bicarbonate pool and catalyzes the dehydration of this pool to CO_2 which then passes into alveolar air. The precise location of the enzyme within the lung remains uncertain but recent investigations indicate that CA is located in or on endothelial cells of the pulmonary capillary bed (Effros *et al.*, 1981; Lonnerholm, 1982). CA in vertebrate red blood cells is also known to play an important role in the CO_2 reactions occurring in the blood. However, the quantitative roles of red blood cell and pulmonary CA have not been clearly established (Crandall *et al.*, 1980; Ponte and Purves, 1980).

It is intriguing that so many different kinds of animals have developed similar solutions to the problem of excreting CO_2, a gas which reacts (hydration-dehydration) slowly with its aqueous environment. The dynamics of the process have been successfully elucidated in mammals using artificially perfused lungs (Crandall and O'Brasky, 1978; Klocke, 1978; Effros *et al.*, 1980). To my knowledge, the perfusion of gill tissues in aquatic organisms for purposes of studying CO_2 excretion is limited to a single study on rainbow trout (Haswell and Randall, 1978). In this paper I will detail the techniques for artificially perfusing gills isolated from a crab and present evidence which supports the hypothesis that crab gill CA plays a direct role in dehydrating the hemolymph bicarbonate pool to CO_2, thus facilitating carbon dioxide removal.

GILL PERFUSION

Crustacean gills have often been used to study transepithelial transport phenomena (Croghan *et al.*, 1965; Mantel, 1967; Tullis, 1973; Berlind and Kamemoto, 1977; Cantelmo, 1977; Pequeux and Gilles, 1978). In all cases the methods used were either inadequately described or produced unphysiological flow patterns of saline through the gills and water across the gills. In no case were the gases of the perfusion medium adjusted to physiological pressures, nor was there an accounting for the leaks around the cannulae or verification of the metabolic viability of the perfused gill.

The animal I chose to establish gill perfusion procedures was the crab *Cancer anthonyi* Rathbun. I chose this crab because it is large and has large gills, is an osmotic and ionic conformer, and is locally abundant. Crabs were purchased from local seafood markets and maintained in either running sea water at ambient temperature (12–

TABLE 1. *Composition of internal perfusate.*

	mM		mM
Na^+	526	Taurine	0.3
K^+	12	Aspartate	0.05
Ca^{++}	24	Threonine	0.1
Mg^{++}	19	Serine	0.18
NH_4^+	0.5	Glutamate	0.02
		Proline	1.0
Cl^-	500	Glycine	0.7
SO_4^{--}	50	Alanine	0.4
HCO_3^-	5	Valine	0.06
		Methionine	0.01
Urea	0.5	Isoleucine	0.05
Glucose	0.65	Leucine	0.05
HEPES	50	Tyrosine	0.02
		Phenylalanine	0.04
		Lysine	0.05
Crab hemolymph	1%	Histidine	0.02
		Arginine	0.1

TABLE 2. *The buffering properties of 3 individual male* Cancer anthonyi *are compared with that of internal perfusate by incubation of samples at different P_{CO_2}'s and measuring pH and C_{CO_2} (15°C).*

	P_{CO_2} (torr)	pH	C_{CO_2} (mM)
Crab #1	1.5	7.811	6.18
Crab #2	1.5	7.849	8.59
Crab #3	1.5	7.853	9.51
Internal perfusate	1.5	7.789	6.46
Crab #1	3.76	7.599	7.32
Crab #2	3.76	7.615	8.63
Crab #3	3.76	7.769	10.15
Internal perfusate	3.76	7.643	7.28
Crab #1	6.01	7.525	8.16
Crab #2	6.01	7.533	8.95
Crab #3	6.01	7.683	11.55
Internal perfusate	6.01	7.533	8.16

16°C) or held in large aquariums (15 ± 1°C) and fed frozen smelt three times each week.

Composition of perfusates

Sea water served as the external perfusate in all experiments. The composition of the internal perfusate (Table 1) approximates that of crab hemolymph. Hemolymph concentrations of the major ions were assumed to be similar to those of *Cancer magister* (Hunter and Rudy, 1975) acclimated to 35‰ sea water. Hemolymph urea was measured by converting urea to ammonia (using urease) and then assaying for ammonia (Sigma technical bulletin no. 640). Hemolymph ammonia was quantified using the same technique but without treating the sample with urease. Amino acid content was determined on two hemolymph samples by Mr. Stan Kulovich in the Amino Acid Lab at the University of California, San Diego. Hemolymph glucose concentration was determined colorimetrically (Sigma technical bulletin no. 510).

The osmotic concentration of the perfusates was determined routinely using a Wescor 5100C vapor pressure osmometer and compared with values measured in *C. anthonyi* hemolymph. The mean osmotic concentration of hemolymph was 1,008 mOsm/kg ± 10.0 SE, n = 10. The mean osmotic concentration of the internal perfusate was 1,030 mOsm/kg ± 8.8 SE, n = 11, while that of the external perfusate was 986 mOsm/kg ± 5.9 SE, n = 9.

The perfusate was buffered with 50 m*M* HEPES (Calbiochem-Behring). The buffering properties of the internal perfusate were found to be similar to that of crab hemolymph incubated at different P_{CO_2}'s (Table 2).

Freshly drawn crab hemolymph was gently bubbled with N_2 for at least 30 min to induce clotting. The sample was then centrifuged to precipitate the clot and a portion of the supernatant was added to the internal perfusate to achieve a final concentration of 1%. This procedure was found to be necessary to prevent the perfused gill from developing a pale blue color several hours into an experiment.

The external perfusate was well aerated, having a pH of 8.1 and a total CO_2 content (C_{CO_2}) of 1.9 m*M*. pH was measured using a Radiometer PHM72 Acid-Base Analyzer and E5021a gun electrode. C_{CO_2} was measured using the method of Cameron (1971). The internal perfusate was gassed with mixtures of O_2 and CO_2 delivered by Wösthoff gas mixing pumps to yield a P_{O_2} of 34 torr and a P_{CO_2} of 2.2 torr. The pH of the perfusate was adjusted to 7.5–7.8. These values approximate those found in the hemolymph of other *Cancer* species (McMahon *et al.*, 1979; deFur, 1980).

TABLE 3. *The carbonic anhydrase activity of gill homogenates of* Cancer anthonyi.*

Gill number	Specific activity (mg^{-1})
1	0.201
2	0.441
3	0.338
4	0.358
5	0.334
6	0.255
7	0.253
8	0.312
6 ($+10^{-4}$ *M* Diamox)	0

* Activity is expressed as

$$\frac{\left(\frac{\text{uncatalyzed time}}{\text{catalyzed time}}\right) - 1.0}{\text{mg protein}}$$

after Burnett et al. (1981*b*) and represents the mean activity of homogenates in two crabs.

In all cases gases were delivered to the gill perfusion apparatus (described below) using Viton tubing (Cole-Parmer). This tubing was preferred over Tygon since it was found that Tygon is oxygen permeable and results in elevated perfusate P_{O_2}.

Gill carbonic anhydrase activity

Gill homogenates were prepared from two individual *C. anthonyi* and assayed for CA activity using the method described by Burnett *et al.* (1981*b*). All gills demonstrated CA activity and the activity of gill 6, used in the perfusion experiments, was completely inhibited by treatment with 10^{-4} *M* Diamox (Table 3).

Assessment of metabolic viability

The metabolic viability of perfused gills was assessed by measuring gill ATP content at the end of a perfusion experiment. Gill ATP content was measured by first plunging the excised gill into liquid N_2, the gill reaching the temperature of liquid N_2 in 15–20 sec. The frozen gill was then homogenized (Polytron, Brinkman Instr.) in 2 ml of ice-cold distilled water. One ml of the homogenate was immediately added to 1 ml ice-cold 12% TCA, mixed, placed on ice for 5–10 min and centrifuged to yield a clear supernatant. The supernatant was assayed for ATP according to Sigma technical bulletin no. 366-UV. A sample of the homogenate was also assayed for protein (Biorad Protein Assay). Gill ATP content was expressed as μmol ATP/mg protein.

I performed several experiments to establish the efficacy of this measurement as an index of metabolic viability. First, I determined that the ATP content of excised gills declines with time. The ATP content of gills removed from a crab and assayed immediately was compared with the ATP content of excised gills incubated for 2 and 3 hr in aerated sea water (15°C) and gills held in nonaerated sea water (4°C) in a gas permeable plastic vial for 24 hr (Table 4). Gill ATP content remains unchanged for 2–3 hr but declines after 24 hr at 4°C. Second, I established that gill ATP content is sensitive to the presence of O_2 in the incubation medium. The ATP content of gills incubated for 2 hr in N_2 bubbled sea water is much lower than gills held similarly in aerated sea water (Table 4). Two other sets of gills treated identically but incubated for an additional hour in aerated sea water indicated that when gills are again provided with oxygen, ATP content increases. Since I also used Diamox in my experiments on perfused gills, I incubated excised gills in sea water containing 4×10^{-4} *M* Diamox for 1 and 2 hr and found no difference between these gills and those treated similarly but without Diamox (Table 4).

From these results I felt that knowledge of gill ATP content provided a good index of metabolic viability. Gills perfused for 5–7 hr routinely had high ATP contents (Table 4).

Gill perfusion chamber and cannulation procedures

Cancer anthonyi has eight pairs of gills which were easily removed and placed in individual containers of ice-cold sea water. In the experiments described in this study, gill 6 (counting from the anterior) was perfused.

The afferent and efferent vessels of gill 6 were cannulated using short lengths of PE tubing held in place with cyanoacrylate

TABLE 4. *Gill ATP content is a function of time and the presence of oxygen in the ambient medium and is independent of the presence of 4 × 10^{-4} M Diamox in the ambient medium. The ATP content of gills perfused for different lengths of time is also shown.*

	Gill number	μmol ATP/mg protein		Gill number	μmol ATP/mg protein
Gills removed from a crab and assayed immediately	5R	0.0113	Gills incubated in ice cold SW (Control) or SW with Diamox (+Diamox)		
	6R	0.0185	Control 1 hr	7R	0.0101
	7R	0.0173		8L	0.00614
	8R	0.0107	+ Diamox 1 hr	7L	0.0118
2 hr in air bubbled SW	6R + 8R	0.0101		8R	0.0117
2 hr in N_2 bubbled SW	6L + 8L	0.0031	Control 2 hr	8R	0.0086
2 hr air → 1 hr air	5R + 7R	0.0140		7L	0.0089
2 hr N_2 → 1 hr air	5L + 7L	0.0092	+ Diamox 2 hr	8L	0.0094
24 hr in SW at 4°C	5L	0.0030		7R	0.0148
	6L	0.0043	Gills perfused for different lengths of time; time from dissection to end of perfusion (hr)*		
			5	6R	0.0290
			5	6L	0.0290
			6	6L	0.0105
			7	6R	0.0195

* Time includes 0.5–1 hr for gill cannulation.

glue (Krazy Glue). The afferent vessel was cannulated first and the gill flushed with internal perfusate for 5–10 min driven by a Masterflex peristaltic pump. This procedure removed residual hemolymph from the gill and in no case did I ever observe hemolymph clotting within the gill. The efferent cannula was then positioned and glued into place. The two cannulae were clamped together to prevent either cannula from twisting and pulling out of the gill. The gill was then picked up by its cannulae and positioned in the perfusion chamber.

The gill perfusion chamber consisted of a Plexiglas block into which a central chamber is carved conforming approximately to the contours of a typical gill (Fig. 1). Small holes were drilled into the central chamber to provide the underside of the gill (efferent vessel side) with a stream of sea water. Holes placed similarly on the opposite side drained the central chamber. The gill and cannulae were sealed into the central chamber with dental wax (Lactona Surgident). A suction was placed on the central chamber using a Masterflex peristaltic pump causing sea water to flow past the gill. The negative pressures in the central chamber were monitored with a manometer and adjusted to values known to exist in the branchial chambers of crabs (−2 to −4 cm water; McMahon and Wilkens, 1983). The pressure used to drive the internal perfusate through the gill was also monitored and found to be between 2 and 8 cm water depending upon the flow rate.

The flow rates of internal and external perfusates were easily controlled. However, "resting" flow rates for both perfusates were calculated from known values for cardiac output and ventilation rate in this species. Gill 6 was assumed to receive 10% of cardiac output and 10% of the ventilation volume since it accounted for 10% of the wet weight of the 16 gills. The values for cardiac output and ventilation rate were 100 ml/kg-min and 600 ml/kg-min, respectively (Burnett *et al.*, 1981*a*).

The perfusates, the tubing carrying perfusate to the gill and perfusion chamber were held at constant temperature (±0.5°C). Experiments were carried out between 15 and 18°C.

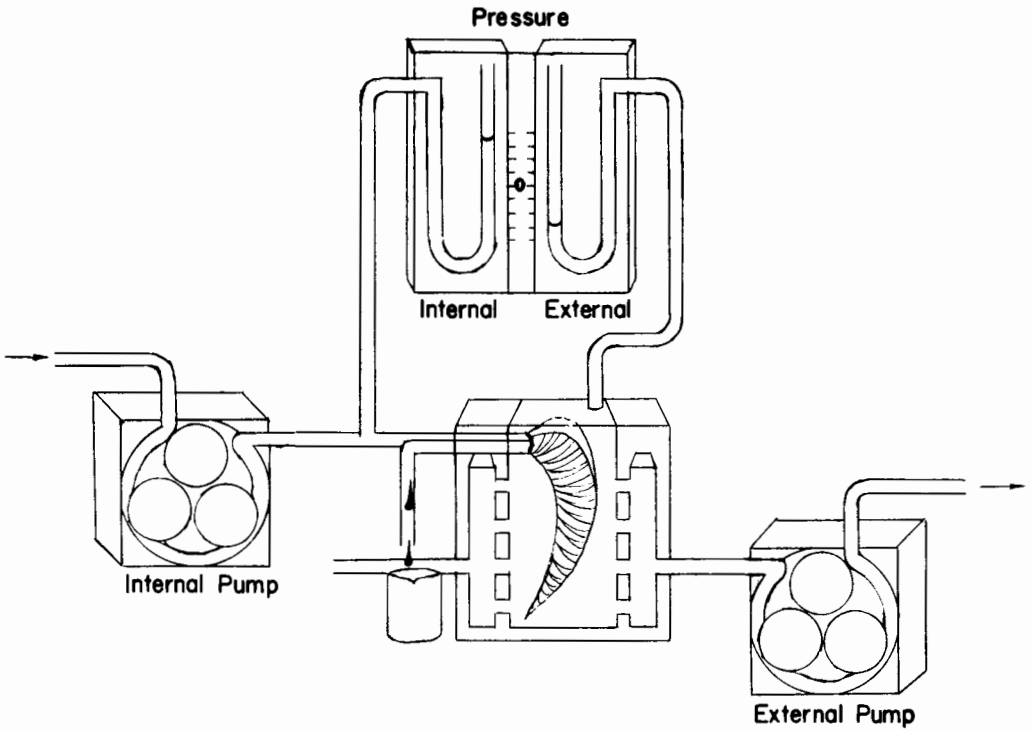

FIG. 1. The gill perfusion apparatus. Internal perfusate is pumped from a reservoir (not shown) by a peristaltic internal pump through the gill at pressures between 2 and 8 cm H_2O and collected for analysis. External perfusate is pumped from a reservoir (not shown) across the external surfaces of the gill by an external pump such that pressures in the gill chamber are −2 to −4 cm H_2O. Perfusion pressures are monitored continuously using manometers.

Leak tests and assessment of internal and external perfusion

Leaks of the internal perfusate around the cannulae into the external perfusate were detected using two methods. In one method [^{3}H]inulin (New England Nuclear) was added to the internal perfusate and the gill perfused internally for 10 min with no external perfusion. Significant accumulation of [^{3}H]inulin in the external perfusate resulted only when a leak was present. Routinely preparations leaked only 1–2% of the radiolabel entering the gill during the 10 min, however, the accumulation of this quantity of radiolabel in the external perfusate was entirely accounted for by gill permeability to the radiolabel.

While the above method provided an accurate measure of small leaks around the cannulae, it was not a convenient on-line measure. An on-line measure of leakiness was provided by monitoring the volume of internal perfusate exiting the gill at high and low negative external perfusate pressures. If a leak appears anywhere in the gill, the volume of internal perfusate leaving the gill is reduced. The presence of a leak can be confirmed by increasing the negative pressure of the external perfusate and noting a corresponding drop in the flow of the internal perfusate.

Good external perfusion of the lamellae was confirmed by adding a small quantity of crystal violet to the external perfusate, perfusing the gill for a short time, then perfusing with fresh external perfusate (containing no crystal violet) and visually inspecting the lamellae for the uptake of stain. Lamellae appeared to be uniformly stained at all points along the gill.

The efficacy of internal perfusion was assessed prior to the termination of each

experiment by adding a small amount of carmine red to the internal perfusate, perfusing the gill for 1–2 min and then visually inspecting the gill for the presence of carmine. Carmine particles would ordinarily not pass through the lamellae, becoming trapped on the afferent side. Good internal perfusion, including the tip of the gill, was routinely accomplished.

Measurement of CO_2 efflux across the gill

The efflux of carbon dioxide from the internal to the external perfusate was followed as ^{14}C. Ten μCi [^{14}C]$NaHCO_3$ (New England Nuclear) was added to approximately 500 ml of the internal perfusate which had previously been pH adjusted and gassed with appropriate mixtures of CO_2 and O_2. The mixture was allowed to stand for at least 30 min before perfusion experiments were performed to ensure equilibration of the label between bicarbonate and dissolved CO_2. In a typical experiment pre- and postgill internal perfusate was sampled and the presence of ^{14}C quantified using liquid scintillation counting techniques. The scintillation fluid was Beta Blend (WestChem Products) and the counter a Beckman LS-133 Liquid Scintillation System.

The gas atmosphere above the internal perfusate was maintained at the original pressures throughout an experiment. Relatively few ^{14}C counts were lost from the perfusate to the gaseous phase during this time.

In some experiments either bovine carbonic anhydrase (0.4 mg/ml) or acetazolamide (Diamox, Lederle; 4×10^{-4} *M*) was added to the internal perfusate. Diamox was also added to the external perfusate to a final concentration of 4×10^{-4} *M*, a concentration greater than that needed to fully inhibit CA activity *in vitro* (Table 3).

EFFECTS OF EXTERNAL PERFUSION FLOW RATE

One of the first experiments I chose to do was to test the effect on CO_2 excretion of various external perfusion flow rates which simulate naturally occurring changes in ventilation rates of crab gill chambers. I was also interested in determining if CO_2 excretion in perfused gills is similar to (calculated) values for CO_2 production in whole animals reported in the literature.

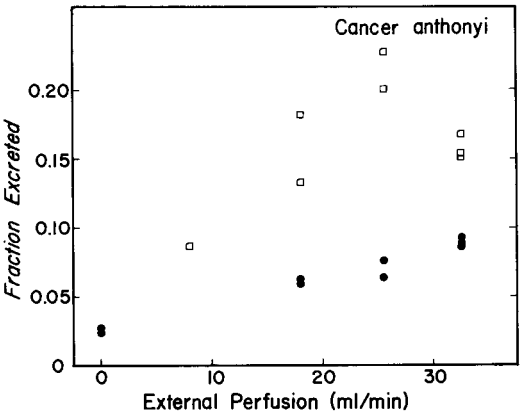

FIG. 2. The fraction of ^{14}C label (as HCO_3^- and CO_2) excreted from the internal perfusate of a perfused gill is a function of the external perfusion flow rate in an untreated gill (closed circles). The addition of bovine carbonic anhydrase (0.4 mg/ml) to the internal perfusate greatly increases the fraction of ^{14}C label excreted (open squares). The variables for the internal perfusate of the untreated gill are pH = 7.86 and C_{CO_2} = 4.69 m*M*; for the CA treated internal perfusate pH = 7.84 and C_{CO_2} = 5.04 m*M*. Flow rate of internal perfusate = 3.1 ml/min. Weight of crab from which gill was excised was 338 g.

The measured changes in CO_2 efflux at different external perfusion rates were as expected (Fig. 2). High external perfusion flow rates increased CO_2 efflux and low rates decreased efflux. Interestingly, significant CO_2 excretion was observed when the perfusion chamber was not ventilated at all. I think this result was due to label effluxing from the internal perfusate as it passed through the large "marginal" channel observed in other crab species (Taylor and Butler, 1978; Taylor and Greenaway, 1979) and may provide the CO_2 in the internal perfusate with access to the large CO_2 sink surrounding the gill.

A value of 20 ml/min approximates the resting external perfusion rate for this particular gill (Fig. 2; crab weight = 338 g). At this flow rate about 0.05 of the CO_2 entering the gill is excreted. This figure can be applied to an equation to predict whole animal CO_2 production in *C. anthonyi*

using a range of values measured for total CO_2 content and known cardiac output.

$$\frac{0.05\ CO_2}{\text{excreted}} \times \frac{5\text{–}10\ \mu\text{mol}\ CO_2}{\text{ml}} \times \frac{100\ \text{ml cardiac output}}{\text{kg-min}} = 25\text{–}50\ \frac{\mu\text{mol}\ CO_2}{\text{kg-min}}$$

The range of predicted CO_2 production agrees well with the range of values calculated from measurements of oxygen uptake in crabs at a similar temperature (McMahon and Wilkens, 1983) and assuming a respiratory quotient between 0.7 (Herreid *et al.*, 1979) and 2 (Henry, 1981).

Effects of Carbonic Anhydrase and Diamox Treatment

The addition of bovine CA to the internal perfusate greatly increases the fraction of radiolabel excreted at several external perfusion flow rates (Fig. 2). This result suggests that the interconversion between bicarbonate and dissolved CO_2 is rate limiting in the excretion process. It also suggests that the direction of conversion favors the dehydration of bicarbonate. The dehydration reaction must be favored since a two- to fourfold increase in CO_2 excretion can hardly be explained by increasing the hydration of the very small dissolved CO_2 pool which is only $\frac{1}{50}$th the size of the large bicarbonate pool. This conclusion is in agreement with that reached by Aldridge and Cameron (1979) who used a computer model to simulate the movement of CO_2 across crab gills.

The large hemolymph bicarbonate pool appears to have access to CA normally present in a gill. Diamox treatment results in a rapid decrease in the flux of radiolabel (Fig. 3). If the variables measured for this system are modeled into the Aldridge and Cameron (1979) model, it is possible to predict the fraction of CO_2 which should be excreted. The possibility that the perfusate CO_2 system is in disequilibrium before it enters the gill is avoided since perfusates were gassed for at least one hour before perfusion was initiated. Aldridge and Cameron (1979) suggested that a major

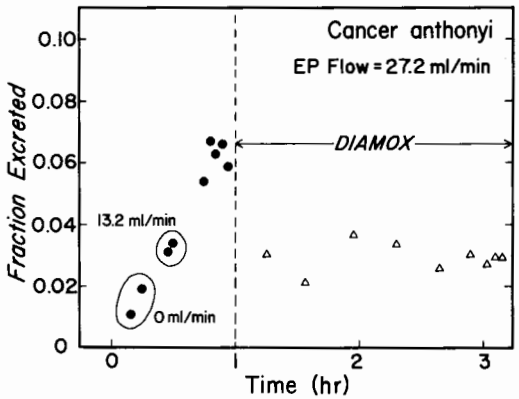

FIG. 3. The fraction of ^{14}C label (as HCO_3^- and CO_2) excreted from the internal perfusate in an untreated perfused gill at several different external perfusion flow rates and at various times (closed circles). Also shown is the effect of Diamox on ^{14}C label excretion when the external perfusion flow rate is 27.2 ml/min (open triangles). Diamox (4×10^{-4} *M*) was added to both the internal and external perfusates. The variables for the internal perfusate of the untreated gill are pH = 7.54 and C_{CO_2} = 3.35 m*M*; for the Diamox treated perfusate pH = 7.56 and C_{CO_2} = 4.05 m*M*. Flow rate of internal perfusate = 3.15 ml/min. Weight of crab from which gill was excised was 566 g. This experiment was duplicated with the same results.

problem in applying the model to *in vivo* systems is our lack of knowledge of *in vivo* P_{CO_2} in crab hemolymph. They suggested that hemolymph is not in equilibrium when it is sampled, *i.e.*, it possesses a higher P_{CO_2}, but that it comes into equilibrium in the measuring apparatus in less time than it takes to make a measurement, *i.e.*, P_{CO_2} declines. The *in vitro* gill perparation avoids this difficulty but I will return to this point later.

Using pH and C_{CO_2} variables corresponding to data presented in Figure 3, the Aldridge and Cameron (1979) model predicts that the untreated gill should excrete 3.12% of its CO_2 and the Diamox treated gill should excrete 2.85%. The values are slightly different due to the slightly different values for pH and C_{CO_2}. The predicted excretion is remarkably close to the measured excretion of radiolabel in the Diamox treated gill (2–3%). The untreated gill, however, was shown to excrete approximately twice this quantity of radiolabel. The only significant differences in the

model between the treated and untreated gills are the rate constants for the hydration-dehydration reactions of CO_2. The untreated gill requires that rate constants for the hydration-dehydration reactions (k_1 and k_2 in the model) be 20–30 times rate constants for uncatalyzed reactions. Calculation of rate constants using known velocities for CA catalyzed reactions reveals that this can easily be the case. Velocities for the dehydration reaction measured in gill homogenates of *Callinectes sapidus* at 15–25°C and pH 7.5–7.8 are 150–250 μmoles $CO_2 \times min^{-1} \times mg\ protein^{-1}$ (Henry and Cameron, 1982). Assuming that crab gill CA has a molecular weight of roughly 30,000 (Woodson and Burnett, unpublished data) and that 25% of the protein in the gill homogenate is CA (probably an overestimate), then the rate constant for the dehydration reaction, k_2, can be estimated according to the following equation (Liébecq, 1971) where E

$$\frac{d[CO_2]}{dt} = k_2[E]$$

$$\text{or} \quad k_2 = \frac{d[CO_2]}{dt[E]} = \frac{200\ \mu\text{mol } CO_2}{\text{min } 0.25\ \text{mg protein}} \times \frac{30{,}000\ \text{g}}{\text{mole}}$$

$$= 400\ \text{sec}^{-1}$$

is the CA concentration. If CA makes up less than 25% of the homogenate protein, then k_2 will be greater. This value of k_2 falls well within the expected values for those predicted in the untreated gill.

The above evidence strongly supports the hypothesis of a gill CA available to the bicarbonate pool in the hemolymph of crabs. Such a gill enzyme must be located on or near the basal membrane of the gill epithelium where it would have ready access to the hemolymph. A potential criticism of the cell surface localization of the enzyme would be the inhibition of enzyme activity by the high hemolymph concentrations of chloride ions (500 m*M*) which are known to inhibit mammalian CA activity (Maren, 1967). However, Henry and Cameron (1982) have determined that crab gill CA retains much of its activity in the presence of 500 m*M* NaCl. CA may also be located intracellularly which would require a bicarbonate permeable basal membrane, similar to the scheme proposed by Haswell and Randall (1978). At the present time neither cell surface nor intracellular localization can be eliminated.

The disequilibrium status of the hemolymph

In light of the above consideration, I think some tentative suggestions regarding the disequilibrium status of the hemolymph can now be made. To do this I will again use the Aldridge and Cameron (1979) gill model and data on *Callinectes sapidus,* since the model was developed for this organism. Henry (1981) provides hemolymph pH and C_{CO_2} data on crabs acclimated to sea water before and after exposure to Diamox. He also measures whole animal CO_2 production before and after Diamox treatment. Using these values and calculating P_{CO_2} before and 6 and 12 hr post-Diamox treatment, CO_2 production can be predicated using the model (Table 5). Pretreatment CO_2 production was predicted from the model twice, using rate constants for both uncatalyzed and catalyzed hydration-dehydration reactions. Comparing the predicted CO_2 production with that actually measured, I find an uncatalyzed production 30% of the actual and a catalyzed production 75–92% of the actual. The 30% value is much smaller than that originally predicted by Aldridge and Cameron (1979) because they assumed a CO_2 production much lower than that which Henry (1981 and Table 5) measured. Treatment with Diamox caused small reductions in hemolymph pH and increases in C_{CO_2} and P_{CO_2} (Henry, 1981). The model predicts a CO_2 production at 6 and 12 hr (now using rate constants for the uncatalyzed reactions) 1.4 and 1.7 times the pretreatment values respectively. These values are greater due to the larger P_{CO_2} gradient but are far below the actual CO_2 production values, which are unaffected by Diamox treatment (Table 5). I think this discrepancy between predicted and measured values is a result of a large disequilibrium brought about by Diamox treat-

TABLE 5. *CO_2 production in* Callinectes sapidus *predicted by the Aldridge and Cameron (1979) gill model using hemolymph variables is compared with CO_2 production measured directly.**

	Hemolymph variables			CO_2 production predicted from model (μmol × kg^{-1} × min^{-1})	% of meas.	Measured CO_2 production (μmol × kg^{-1} × min^{-1})
	pH	C_{CO_2} (m*M*)	P_{CO_2} (torr)			
Pre Diamox treatment						
Using rate constants for uncatalyzed reactions	7.79	6.3	2.55	22.9	30.3	75.6
Using rate constants for CA catalyzed reactions				56.3–69.2	74.5–91.5	
6 hr post Diamox treatment						
Using rate constants for uncatalyzed reactions	7.75	8.0	3.57	31.7	44.5	71.2
12 hr post Diamox treatment						
Using rate constants for uncatalyzed reactions	7.71	8.9	4.35	38.3	49.9	76.8

* Values for pH, C_{CO_2} and measured CO_2 production are from Henry (1981). P_{CO_2} is calculated from pH and C_{CO_2} using constants reported by Truchot (1976).

ment. Disequilibrium may also exist in the untreated crabs but it must be much smaller. Henry and Cameron (1982) have measured low levels of CA activity in muscle, heart, branchial epithelium and green gland in this species. It is likely that CA activity in these tissues serves to catalyze the hydration of CO_2 at some point after CO_2 is metabolically produced by the tissue, thus moving the system toward equilibrium. Support for this hypothesis is found in the Diamox induced pH disequilibria in perfused rabbit hind limb and liver which contain CA activity (O'Brasky and Crandall, 1980).

When Diamox is used to inhibit CA activity in all tissues, the catalyzed hydration of CO_2 by enzyme in muscle tissue, for example, is inhibited. Thus CO_2 metabolically produced in crab muscles enters the hemolymph and is hydrated at the slow uncatalyzed rate creating a disequilibrium. This results in actual hemolymph P_{CO_2}'s larger than those calculated. Thus, a larger fraction of the total CO_2 reaches the gill as dissolved CO_2 which is more diffusible (Gutknecht *et al.*, 1977). P_{CO_2} in Diamox treated crabs must be 2–3 times the calculated values to account for the discrepancy between CO_2 production predicted by the model and that measured by Henry (1981). Therefore, the much steeper P_{CO_2} gradients between hemolymph and water and the greater CO_2 disequilibrium in the hemolymph provide an explanation for why Henry (1981) measured no change in CO_2 production when he treated crabs with Diamox.

It is unclear why the strategy of allowing the hemolymph to stay in a state of disequilibrium until it reaches the gill is not followed. Surely this strategy results in the removal of large quantities of CO_2 but without the "work" involved in making an enzyme. Perhaps the answer lies in the greater capacity of an enzyme catalyzed system to keep up in situations when the crab produces more CO_2, *e.g.*, during exercise.

I suggested at the beginning of this article that CA associated with respiratory organs in many different animals serves the important function of mobilizing blood bicarbonate to the more diffusible dissolved CO_2 (Gutknecht *et al.*, 1977) for purposes of CO_2 excretion. I have presented evidence suggesting that crabs employ a similar strategy. This hypothesis is consistent with the well established idea that crab gill CA serves an important ion regulating function. Furthermore, it accounts for the presence of the enzyme in the anterior gills which are not associated with ionic regulation in crabs.

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REFERENCES

Aldridge, J. B. and J. N. Cameron. 1979. CO_2 exchange in the blue crab, *Callinectes sapidus* (Rathbun). J. Exp. Zool. 207:321–328.

Berlind, A. and F. I. Kamemoto. 1977. Rapid water permeability changes in eyestalkless euryhaline crabs and in isolated, perfused gills. Comp. Biochem. Physiol. 58A:383–385.

Burggren, W. W. 1979. Bimodal gas exchange during variation in environmental oxygen and carbon dioxide in the air breathing fish *Trichogaster trichopterus*. J. Exp. Biol. 82:197–213.

Burggren, W. and S. Haswell. 1979. Aerial CO_2 excretion in the obligate air breathing fish *Trichogaster trichopterus*: A role for carbonic anhydrase. J. Exp. Biol. 82:215–225.

Burnett, L. E., P. L. deFur, and D. D. Jorgensen. 1981*a*. Application of the thermodilution technique for measuring cardiac output and assessing cardiac stroke volume in crabs. J. Exp. Zool. 218: 165–173.

Burnett, L. E., P. B. J. Woodson, M. G. Rietow, and V. C. Vilicich. 1981*b*. Crab gill intra-epithelial carbonic anhydrase plays a major role in hemolymph CO_2 and chloride ion regulation. J. Exp. Biol. 92:243–254.

Cameron, J. N. 1971. A rapid method for determination of total carbon dioxide in small blood samples. J. Appl. Physiol. 31:632–634.

Cantelmo, A. C. 1977. Water permeability of isolated tissues from decapod crustaceans—1. Effect of osmotic conditions. Comp. Biochem. Physiol. 58A:343–348.

Crandall, E. D., A. Bidani, and J. E. O'Brasky. 1980. Intravascular end-capillary CO_2-H^+ disequilibrium. *In* C. Bauer, G. Gros, and H. Bartels (eds.), *Biophysics and physiology of carbon dioxide*, pp. 306–314. Springer-Verlag, New York.

Crandall, E. D. and J. E. O'Brasky. 1978. Direct evidence for participation of rat lung carbonic anhydrase in CO_2 reactions. J. Clin. Invest. 62:618–622.

Croghan, P. C., R. A. Curra, and A. P. Lockwood. 1965. The electrical potential difference across the epithelium of isolated gills of the crayfish *Austropotamobius pallipes* (Lereboullet). J. Exp. Biol. 42:463–474.

deFur, P. L. 1980. Respiratory consequences of air exposure in *Cancer productus*, an intertidal crab. Ph.D. Thesis, University of Calgary.

Effros, R. M., R. S. Y. Chang, and P. Silverman. 1978. Acceleration of plasma bicarbonate conversion to carbon dioxide by pulmonary carbonic anhydrase. Science 199:427–429.

Effros, R. M., G. Mason, and P. Silverman. 1981. Asymmetric distribution of carbonic anhydrase in the alveolar-capillary barrier. J. Appl. Physiol. 51:190–193.

Effros, R. M., L. Shapiro, and P. Silverman. 1980. Carbonic anhydrase activity of rabbit lungs. J. Appl. Physiol. 49:589–600.

Gutknecht, J., M.A. Bisson, and F.C. Tosteson. 1977. Diffusion of carbon dioxide through lipid bilayer membranes, effects of carbonic anhydrase, bicarbonate, and unstirred layers. J. Gen. Physiol. 69: 779-794.

Haswell, M. S. and D. J. Randall. 1978. The pattern of carbon dioxide excretion in the rainbow trout *Salmo gairdneri*. J. Exp. Biol. 72:17–24.

Haswell, M. S., D. J. Randall, and S. F. Perry. 1980. Fish gill carbonic anhydrase: Acid-base regulation or salt transport? Am. J. Physiol. 238:R240–R245.

Henry, R. P. 1981. The role of branchial carbonic anhydrase in respiration, ion regulation and acid-base balance in two species of decapod crustaceans: The aquatic species *Callinectes sapidus* and the terrestrial *Gecarcinus lateralis*. Ph.D. Diss., University of Texas at Austin.

Henry, R. P. 1984. The role of carbonic anhydrase in blood ion and acid-base regulation. Amer. Zool. 24:241–251.

Henry, R. P. and J. N. Cameron. 1982. The distribution and partial characterization of carbonic anhydrase in selected aquatic and terrestrial decapod crustaceans. J. Exp. Zool. 221:309–321.

Herreid, C. F., L. W. Lee, and G. M. Shah. 1979. Respiration and heart rate in exercising land crabs. Respir. Physiol. 36:109–120.

Hunter, K. C. and P. P. Rudy. 1975. Osmotic and ionic regulation in the Dungeness crab, *Cancer magister* Dana. Comp. Biochem. Physiol. 51A:439–447.

Klocke, R. A. 1978. Catalysis of CO_2 reactions by lung carbonic anhydrase. J. Appl. Physiol. 44: 882–888.

Laurent, P. and S. Dunel. 1980. Morphology of gill epithelia in fish. Am. J. Physiol. 238:R147–R159.

Lonnerholm, G. 1982. Pulmonary carbonic anhydrase in the human, monkey, and rat. J. Appl. Physiol. 52:352–356.

Liébecq, C. 1971. Introduction to enzyme kinetics. *In* Z. M. Bacq (ed.), *Fundamentals of biochemical pharmacology*, pp. 59–86. Pergamon Press, New York.

Mantel, L. H. 1967. Asymmetry potentials, metabolism and sodium fluxes in gills of the blue crab,

Callinectes sapidus. Comp. Biochem. Physiol. 20: 743–753.

Maren, T. H. 1967. Carbonic anhydrase: Chemistry, physiology, and inhibition. Physiol. Rev. 47:595–781.

McMahon, B. R. and L. E. Burnett. 1981. Acid-base balance and CO_2 excretion in two littoral crabs *Hemigrapsus nudus* and *Pachygrapsus crassipes.* Amer. Zool. 21:1014.

McMahon, B. R., L. E. Burnett, and P. L. deFur. 1984. Carbon dioxide excretion and carbonic anhydrase function in the red rock crab *Cancer productus.* J. Comp. Physiol. (In press)

McMahon, B. R., D. G. McDonald, and C. M. Wood. 1979. Ventilation oxygen uptake and haemolymph oxygen transport, following enforced exhausting activity in the Dungeness crab *Cancer magister.* J. Exp. Biol. 80:271–285.

McMahon, B. R. and J. L. Wilkens. 1983. Ventilation, perfusion and oxygen uptake. *In* L. Mantel (ed.), *Biology of Crustacea,* Vol. 5, Chapter 6. Academic Press, New York.

O'Brasky, J. E. and E. D. Crandall. 1980. Organ and species differences in tissue vascular carbonic anhydrase activity. J. Appl. Physiol. 49:211–217.

Pequeux, A. and R. Gilles. 1978. Osmoregulation of the euryhaline chinese crab *Eriocheir sinensis.* Ionic transports across isolated perfused gills as related to the salinity of the environment. Proceedings of the 12th EMBS, Stirling, Scotland. *Physiology and behavior of marine organisms,* pp. 105–111. Oxford-New York, Pergamon Press.

Ponte, J. and M. J. Purves. 1980. Changes in pH and P_{CO_2} with time in pulmonary post-capillary blood in cats. *In* C. Bauer, G. Gros, and H. Bartels (eds.), *Biophysics and physiology of carbon dioxide,* pp. 315–320. Springer-Verlag, New York.

Randall, D. J. and C. M. Wood. 1981. Carbon dioxide excretion in the land crab (*Cardisoma carnifex*). J. Exp. Zool. 218:37–44.

Taylor, E. W. and P. J. Butler. 1978. Aquatic and aerial respiration in the shore crab, *Carcinus maenas* (L.), acclimated to 15°C. J. Comp. Physiol. 127:315–323.

Taylor, H. H. and P. Greenaway. 1979. The structure of the gills and lungs of the acid-zone crab, *Holthuisana* (*Austrothelphusa*) *transversa* (Brachyura: Sundathelphusidae) including observations on arterial vessels within the gills. J. Zool., London 189:359–384.

Truchot, J. P. 1976. Carbon dioxide combining properties of the blood of the shore crab *Carcinus maenas* (L.): Carbon dioxide solubility coefficient and carbonic acid dissociation constants. J. Exp. Biol. 64:45–57.

Tullis, R. E. 1973. Crustacean CNS factors influencing water movement through gills and intestine. Amer. Zool. 13:1275.

Wolcott, T. G. and D. L. Wolcott. 1982. Urine reprocessing for salt conservation in terrestrial crabs. Amer. Zool. 22:897.